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Estrogen receptor (ER) is a ligand-activated transcription activator. The detailed mechanism of ER-mediated transcription is unclear. We demonstrated that ER-mediated transcription in cell-free transcription system is ligand-dependent. Antiestrogens ICI164,384, ICI182,780 and 4-hydroxytamoxifen significantly inhibited ER-mediated transcription. Estradiol overcame the inhibitory effect of the antiestrogens and induced ER-mediated transcription. Under the condition used for the transcription assay, ICI164,384 and ICI182,780 inhibited ER-ERE complex formation which might contribute to the inhibitory effect of these antiestrogens on ER-mediated transcription. 4-OH TAM changed the mobility of ER-ERE complex which suggests a conformational change of the complex. Steroid receptor coactivator-1 (SRC-1) significantly augmented ER-mediated-transcription *in vitro*, supporting that SRC-1 is involved in ER signal pathway. ER hormone binding domain (HBD) that binds to ER associated proteins (ERAPs) in a ligand-dependent manner in previously studies inhibited ER-mediated transcription *in vitro*. In contrast, truncated ER HBD lacking of 535 to 595 amino acids which binds to estradiol but does not associate with ERAPs did not affect ER-Mediated effect in this study, suggesting that ERAPs are required for the full transactivation activity of ER.

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Hong Liu 12/26/97

PI - Signature Date

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INTRODUCTION

The Estrogen receptor (ER) is a member of nuclear hormone receptor superfamily which includes steroid hormone receptors, thyroid and retinoid hormone receptors, vitamin D receptor, and a large number of so-called orphan receptors for which no ligands have been identified (1, 2, 3, 4). These receptors function as ligand-activated transcription factors. ER was identified in 1960s and the function of ER as a transcription regulator was also proposed (5, 6). Since then, extensive studies have been conducted to probe the detail mechanism of ER-mediated effects at molecular level. With the cloning of ER gene (7, 8), significant progresses have been made in the elucidation of the structure of ER and the dissection of the mechanism of ER-mediated signal transduction. The human ER is a protein of 595 amino acids and has a molecular weight of approximately 67 kDa (7, 8). Like all members of the nuclear hormone receptor superfamily, it has A to F domains from N-terminus to C-terminus and has a structure which includes a C-terminal domain with hormone binding, dimerization and hormone-dependent transcriptional activation (AF2) activities, a hinge region, a highly conserved central DNA binding domain with two zinc fingers, and an N-terminal domain which has autonomous transcription activation activity (AF1) in a cell- and promoter-specific manner. Although different mechanisms have been proposed, most studies on ER-mediated signal transduction have been carried out on the classic ER-ERE pathway in which ER binds to estrogen, forms a homodimer, recognizes and binds to a palindromic cognate estrogen receptor responsive elements (EREs) with a consensus sequence of GGTCANNNTGACC which locates in the regulatory regions of ER targeted genes such as pS2, progesterone receptor, heat shock protein 27 (Hsp27), c-myc, cathepsin D and so on and then regulates expression of these genes (9, 10, 11, 12, 13, 14).

However, the detailed mechanism of ER-mediated transcription is still unknown. There is evidence that ER facilitates the formation of the initiation complex (15). The extensive studies on ER-mediated signal transduction led to the discovery of a set of ER associated proteins (ERAPs) which opens a broad new field for studying nuclear receptors. Up to now, several nuclear receptor associated proteins have been identified or cloned in different laboratories, including

ERAP140 (16), RIP140 (17), SRC-1 and related proteins (18, 19, 20, 21, 22), p300/CBP (23, 24, 25, 26), SNF2 (27), TIF1 (28), and TRIP1 (29). These nuclear receptor associated proteins associate with nuclear receptors in ligand-dependent manners, which correlates to the ligand-dependent transcription of the receptors in cells, suggesting a putative role of the nuclear receptor associated proteins in ligand-activated receptor-mediated transcription. The transcription activation activity of ER has been demonstrated *in vivo* and *in vitro*. It has been showed that SRC-1, RIP140, p300/CBP, SPT6 (30) and two human homologues of yeast SWI2/SNF2 (27) augment estrogen-dependent transcription activity of ER in yeast and mammalian cells.

In this study, a cell-free transcription system is used to study the effects of ER-associated proteins on ER-mediated transcription. We show that ER-mediated transcription *in vitro* is ligand-dependent under the condition used in this study. ER hormone binding domain (HBD) associated proteins are required for the full transcription activity of ER. SRC-1 is involved in ER-mediated transcription *in vitro*.

EXPERIMENTAL PROCEDURES

Plasmids — pAdMLPERE3 is described previously (31) (kindly provided by Dr. C. Abbondanza), which contains three copies of ERE linked to a minimal adenovirus major late promoter (-53 to +9) and a 400 nucleotides of G-less cassette as the reporter. The internal control (pAdMLPmERE) which has the same promoter and a shorter G-less cassette (200 nucleotides) was generated by replacing the three copies of ERE by a mutated ERE (5' - AGGACACAGTGTCCT- 3') which abolishes the formation of ER-ERE complex (32). GST-HBD3, GST-Δ534 are described previously (16). hSRC-1 cDNA that is the equivalent of that reported by Onate et al (18) was subcloned as a glutathione-S-transferase fusion protein (GST-SRC-1) and expressed in E. Coli strain Y1090 strain.

Protein Expression—Human ER is overexpressed in baculovirus system and partially purified by Mono S column (PanVera Inc.). GST-HBD3, GST-Δ534 and GST-SRC-1 are expressed in bacteria and purified on gluthione sepharose (Pharmcia).

In Vitro Transcription Assay—HeLa cells were grown in suspension and nuclear extract was prepared as previously described (33). The *in vitro* transcription reaction contained: 10 mM HEPES (pH 7.9), 8.5% glycerol, 60 mM KCl, 7.5 mM $MgSO_4$, 5 mM creatine phosphate, 2.5 mM DTT, 30 U RNasin inhibitor, 5 mg/ml BSA, 12.5 mM ATP and UTP, 5 μM CTP, 40 μM 3-O'-methyl-GTP, 20 μCi [α-P^{32}]CTP (800 Ci/mmol), 10 U RNase T1. Different concentration of templates (pAdMLPERE3 and pAdMLPmERE) were used. ER was incubated with hormones at 30°C for 20 minutes, then incubated with HeLa nuclear extract (30 - 40 μg) at 30°C for another 20 minutes followed by 30 minute incubation with templates at 30°C. The transcription was initiated by adding NTPs. Reactions were stopped by addition of 200 μl of stopping buffer (10 mM Tris (pH 7.9), 10 mM EDTA, 1 M ammonium acetate, 0.5% SDS, and 70 μg/ml yeast tRNA) and extracted once with phenol/chloroform/isoamyl alcohol (25/24/1/) and once with chloroform/isoamyl alcohol (24/1), precipitated and separated on a 6% sequencing gel. The gel was autoradiographed at -70°C with intensifying screens.

Gel Shift Assay — ERE (5'-GATCTCTTTGATCAGGTCACTGTGACCTGACTTTG-3')

oligonucleotides were annealed and labeled with [α–P^{32}]dGTP (3000 Ci/mmol) by Klenow fragment. ER was incubated under the same condition as that for *in vitro* transcription assay except that p^{32}-ERE was used instead of plasmid templates. The ER-ERE complex was separated on a 4% polyacrylamide gel in 0.5 X TGE (32).

RESULTS

Baculovirus-Expressed Human ER Induces Transcription in Vitro — To study the function of ER associated proteins *in vitro*, we demonstrated that ER induced transcription in this system. Figure 1 shows that the partial purified baculovirus-ER does not affect transcription on the internal control template which contains a mutated ERE site; it does significantly increase transcription from the specific template with three copies of ERE sites in the absence of estradiol (E_2) (Fig. 1 lane 2). As reported previously (15, 31), E_2 does not further increase ER-mediated transcription (Fig. 1 Lane 3).

To demonstrate E_2-activated ER-mediated transcription, we tested the effects of different major components such as the concentration of HeLa nuclear extract, templates and ER on ER-mediated transcription in the cell-free transcription system. The amount of HeLa nuclear extract (30 to 60 μg) did not have significant effect on ER-mediated transcription *in vitro* (data not shown). However, the concentration of the specific template is a critical factor on ER-mediated effect in this system as shown in Figure 2. When high concentration of template such as 100 ng/reaction is used, ER does not stimulate transcription from the ERE-containing template (lane 4 to 6) because of the high basal activity. However, ER significantly increases the level of the specific transcript when 20 ng/reaction or less of the template is used (lane 1 to 3). Figure 3 elucidates a concentration-dependent effect of ER on ER-mediated transcription *in vitro*. With increasing concentration of ER, ER-mediated transcription increases. However, ER does not further stimulate transcription at the concentrations higher than 400 fmol (data not shown), and as the matter of fact the transcription level slightly decreases as shown previously (15).

ER-Mediated Transcription in Vitro is Ligand-Dependent — To study hormone-

dependent effect on ER-mediated transcription *in vitro*, three antiestrogens including 4-hydroxy-Tamoxifen (4-OH TAM), ICI164,384 and ICI182,780 were tested in the system. ICI164,384 and ICI182,780 are pure ER antagonists and 4-OH TAM is a partial ER agonist and antagonist (34, 35). In Figure 4, ER stimulates transcription from the specific template independent of E_2 (lane 2 and 3) as shown earlier. ICI164,384 significantly inhibits ER-mediated transcription in the absence of E_2 in a concentration-dependent manner (compare lane 2 and lane 4 to 6). When exogenous E_2 is added, E_2 overcomes the inhibitory effect of ICI164,384 and induces ER-mediated transcription (land 7 to 9). Similar results are obtained when ICI182,780 (lane 10 to 15) and 4-OH TAM (lane 16 to 21) are used.

How do the antiestrogens inhibit ER-mediated transcription? We investigated the effects of the antiestrogens on ER DNA binding ability. ER-ERE complex migrates faster when ER binds to E_2 than that in the absence of hormone or in the presence of 4-OH TAM (32). In ERE gel mobility shift assay (Fig. 5), E_2 does not alter the migration rate of ER-ERE complex (lane 1 and 2), further suggesting that ER already binds to E_2. ICI164,384 and ICI182,780 inhibit the formation of ER-ERE complex, which might contributes to the inhibitory effects of these two antagonists on ER-mediated transcription. 4-OH TAM does not affect the formation of ER-ERE complex. However, the migration of the ER-ERE complex in the gel is slightly slower when ER binds to 4-OH TAM as seen previously (32), suggesting that 4-OH TAM induces a conformation change of the complex which might affect the communication of ER and basal transcription factors and results in inhibition of ER-mediated transcription.

SRC-1 Is Involved in ER-Mediated Transcription In Vitro— SRC-1 is cloned as a steroid hormone receptor coactivator (18). It might be one of the communicators between ER and the basal transcription factors. To investigate the effect of SRC-1 on ER-mediated transcription *in vitro*, SRC-1 is constructed as a gluthione-*S*-transferase (GST) fusion protein (GST-SRC-1) and expressed in bacteria (see EXPERIMENTAL PROCEDUREs). As shown in Figure 6, GST-SRC-1 slightly increases basal transcription level from the internal control template and the transcription from the specific template in the absence of ER (lane 1, 4, 7, 10 and 13). When

added with ER, GST-SRC-1 significantly increases ER-mediated transcription in a concentration-dependent way (lane 2 and 3, 5 and 6, 8 and 9, 11 and 12, 14 and 15).

The Proteins Which Associate with ER Hormone Binding Domain Are Required for the Full Transcription Activation Activity of ER — There are several proteins found to associate with ER hormone binding domain (HBD) in a hormone dependent manner (16, 17, 18, 23). These proteins bind to ER hormone binding domain in the presence of E_2 and its synthetic analog diethylstilbestrol (DES). They do not associate with ER hormone binding domain when antiestrogens such 4-OH TAM, ICI164,384 and ICI182,780 are present.

To determine the effects of these ER HBD associated proteins on ER-mediated transcription *in vitro*, we used GST-HBD3 that GST fuses to ER HBD (16) as a dominant negative competitor for ER. Figure 7 shows that GST-HBD3 does not affect the basal transcription level, however, it significantly inhibits ER-mediated transcription. In contrast, GST-Δ534 (16) is used as a negative control. GST-Δ534 lacks of 535-595 amino acids of ER, It binds to hormone, but does not bind to ER associated proteins (16). GST-Δ534 does not affect ER-mediated transcription as shown in lane 13 to 15 in Figure 7.

DISCUSSION

ER Induces Transcription in Vitro—It has been reported that ER induces transcription *in vitro* using purified calf uterus ER (31) and baculovirus expressed ER (15). In both studies, ER-mediated transcription was hormone-independent. Under certain salt and temperature condition which affects ER-ERE complex formation, ER-mediated transcription *in vitro* was estradiol-stimulated (36). The results shown in Figure 1 to figure 3 also indicate that ER-mediated transcription *in vitro* is E_2-independent under the condition used. E_2-independent ER-mediated transcription *in vitro* does not correlate with the *in vivo* data that ER is a ligand-dependent transcription factor, suggesting that the ER is somehow activated during the purification procedure or there are estrogen-like activities in the ER fraction or HeLa nuclear extract, since the Sf9 cells used for expressing human ER and HeLa cells were not grown in hormone-free condition. By using antiestrogens we demonstrated that partially purified baculovirus-expressed

human ER induces transcription *in vitro* in a hormone-dependent manner. In the absence of antiestrogens (Fig. 4, lane 2 and 3), ER-mediated transcription is independent of E_2 as reported previously. However, Antiestrogens ICI164,384, ICI182,780 and 4-OH TAM inhibited ER-mediated transcription (Fig. 4). According to previous study (32), E_2 speeds the migration of ER-ERE complex in ERE mobility shift assay. In figure 5 (Lane 1 and 2), E_2 did not alter the ER-ERE complex which suggests that the partially purified ER bound to estrogen already, explaining why ER-mediated transcription is E_2-independent in the absence of antiestrogens. ICI164,384 and ICI182,780 which are pure antiestrogens inhibit the formation of ER-ERE complex and inhibit ER-mediated transcription. Partial ER antagonist 4-OH TAM changes the migration of the ER-ERE complex and inhibits ER-mediated transcription to less extend. E_2 overcomes the inhibitory effects of the antiestrogens and induces ER-mediated transcription.

SRC-1 Enhances ER-Mediated Transcription in vitro—SRC-1 has been reported as a steroid hormone receptor coactivator (18). It has been demonstrated that SRC-1 augments steroid receptor-mediated transcription *in vivo* (18, 37, 38, 39). The recent study showed that the function of SRC-1 is essential for the transactivation activity of progesterone receptor *in vitro* (40). In this study, we demonstrated that SRC-1 enhanced ER-mediated transcription *in vitro*.

SRC-1 is only one of the steroid hormone receptor coactivators. To study how significant SRC-1 in ER-mediated signal pathway, we need determining the ER-mediated transactivation activity in SRC-1 deficient system which can be obtained by immunodepleting SRC-1 using SRC-1 specific antibody. At current stage, we have the technical problems to do such a experience. Whether are the ERAPs required for ER-mediated transcription? We were planning to use ERAP-deficient system to study the functions of the ERAPs. We tried to deplete ERAPs from the transcription system using GST-HBD affinity column in the presence of E2. However, it was very difficult to deplete ERAPs from the system completely (data not show). Instead, we added GST-HBD into the transcription system as the competitor for ER. Our results (Figure 7) showed that GST-HBD inhibited ER-mediated transcription in vitro. On the other hand, GST-Δ534 which did not bind to ERAPs did not block ER-mediated effect. This piece of data

suggested that ERAPs are required for the full transactivation activity of ER.

According to the Statement of Work outlined in the proposal, the project progresses ahead of the schedule. Next I like to study the effects of different antiestrogens in ER-mediated transcription *in vitro*.

Acknowledgments—We are grateful to C. Abbondanza for the generous gift of the pMLERE plasmids, Dr. C. Glass for the hSRC-1 cDNA, PanVera Inc. for providing the partially purified baculovirus-hER.

CONCLUSIONS

In this study, we demonstrated that ER-mediated transcription *in vitro* is ligand-dependent. Antiestrogens such as 4-OH TAM, ICI 164,384 and ICI 182,780) significantly inhibited ER-mediated effect, and E_2 overcame the inhibitory effects of antiestrogens. SRC-1 was involved in ER-mediated transcription *in vitro*. ERAPs associated with HBD of ER are required for the full activation of ER. Since ERAPs are very important in ER-mediated transcription, they might be therapeutic targets for designing new anti-breast cancer drugs.

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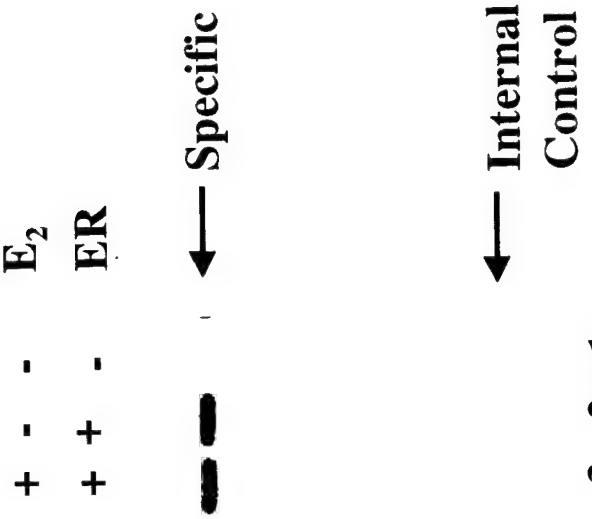
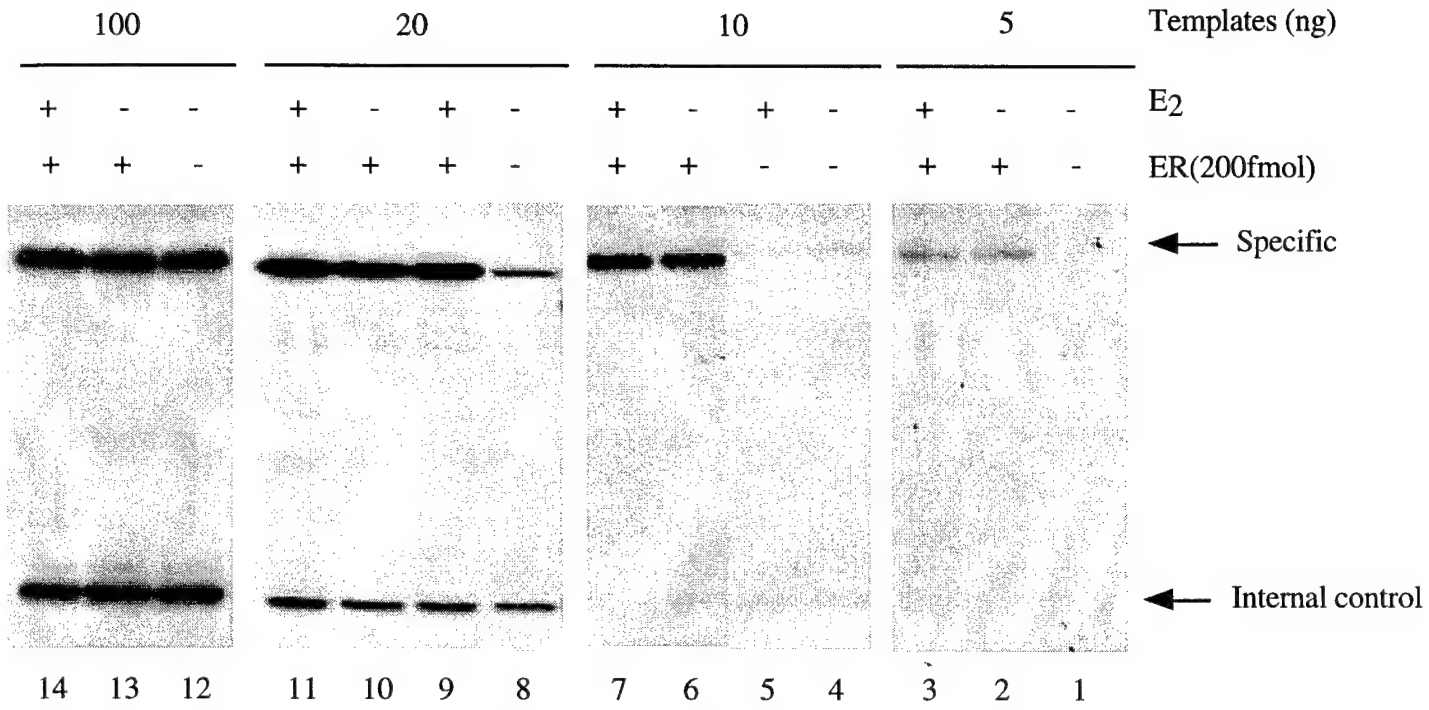
Figure 1

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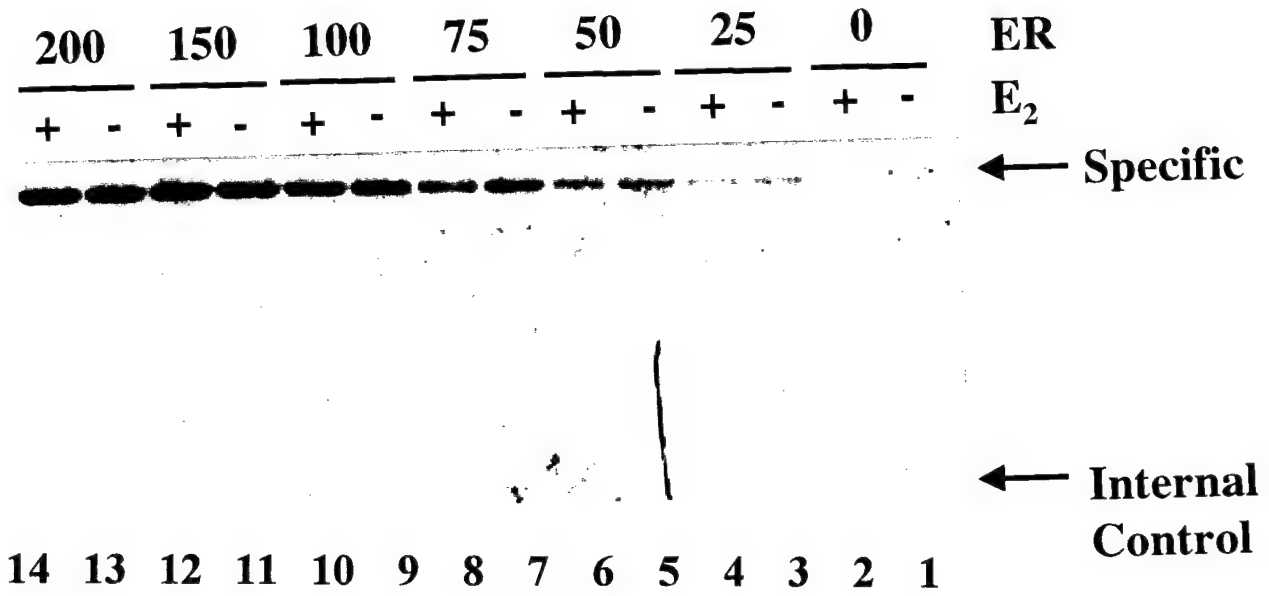
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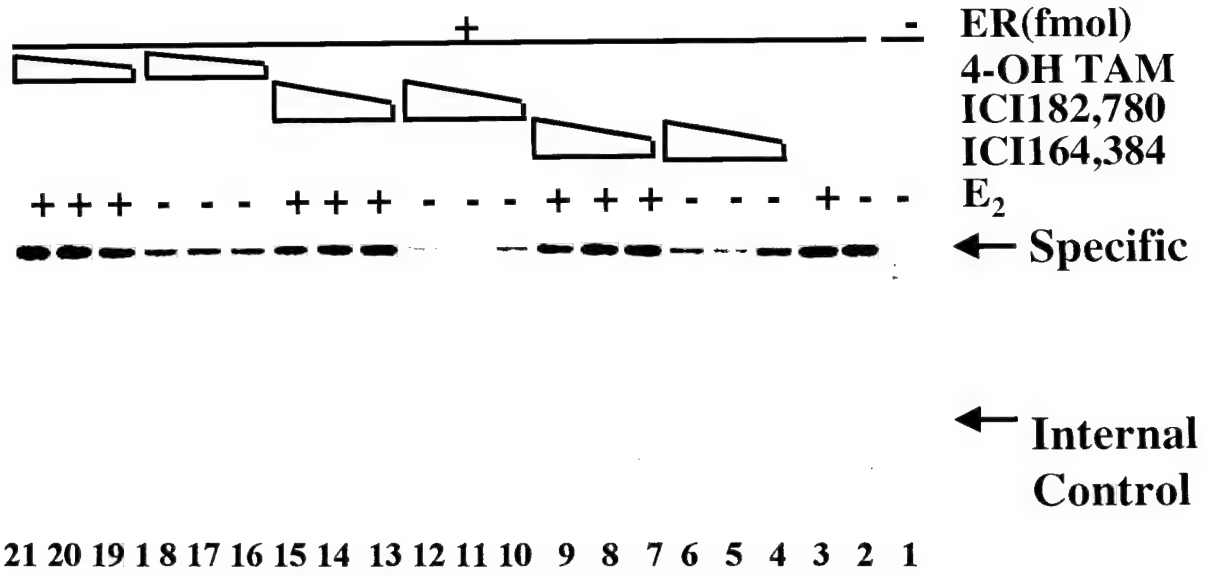
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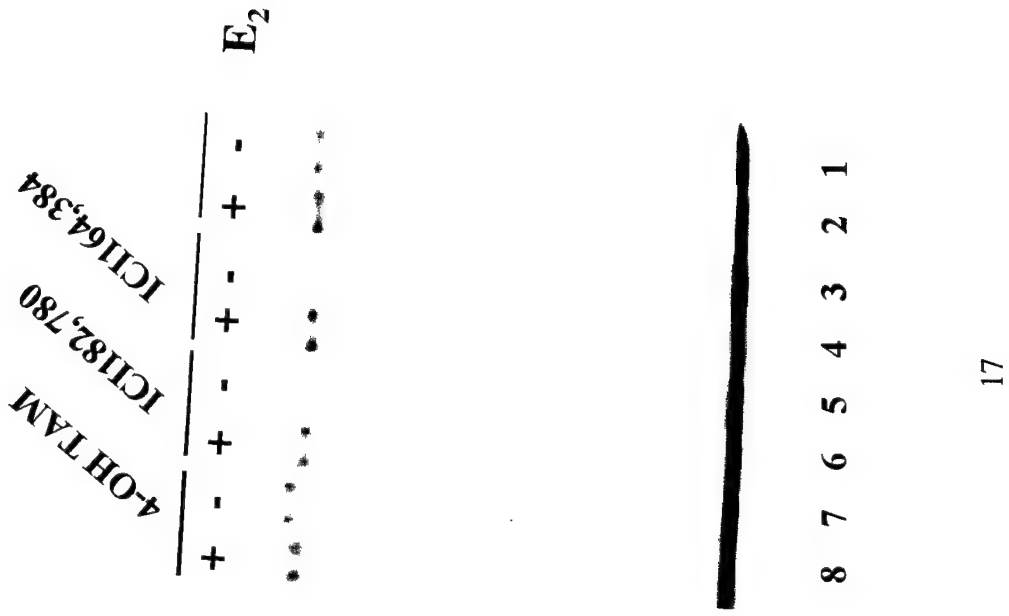
Figure 4

Figure 5



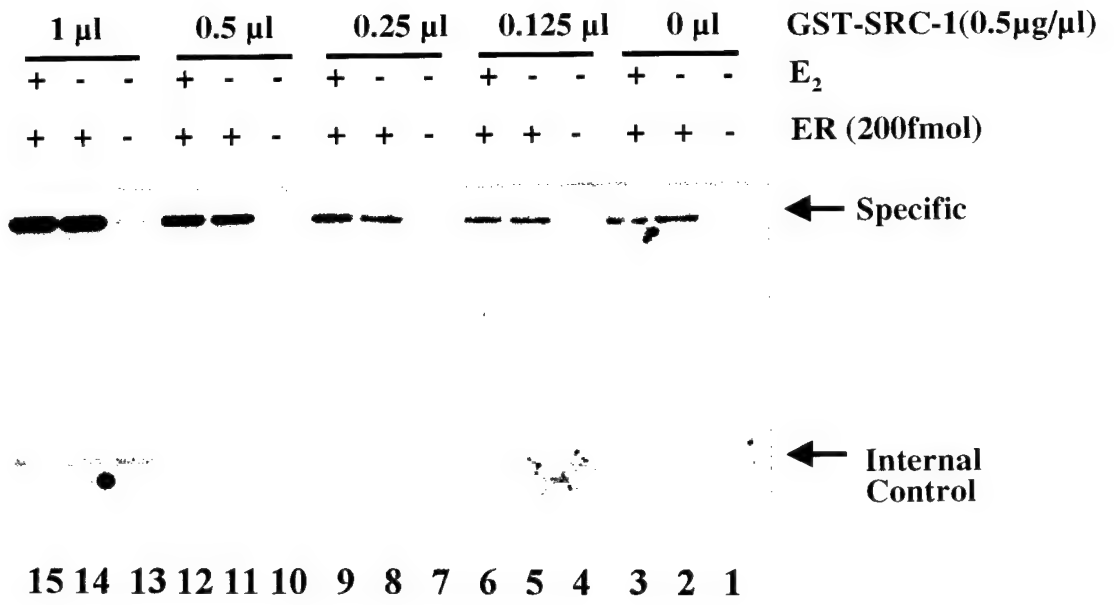

1 µl
0.5 µl
0.25 µl
0.125 µl
0 µl
GST-SRC-1(0.5µg/µl)
+ - - + - - + - - + - - + - - E2
+ + - + + - + + - + + - + + - ER (200fmol)
Specific
Internal Control
15 14 13 12 11 10 9 8 7 6 5 4 3 2 1

Figure 6

Figure 7

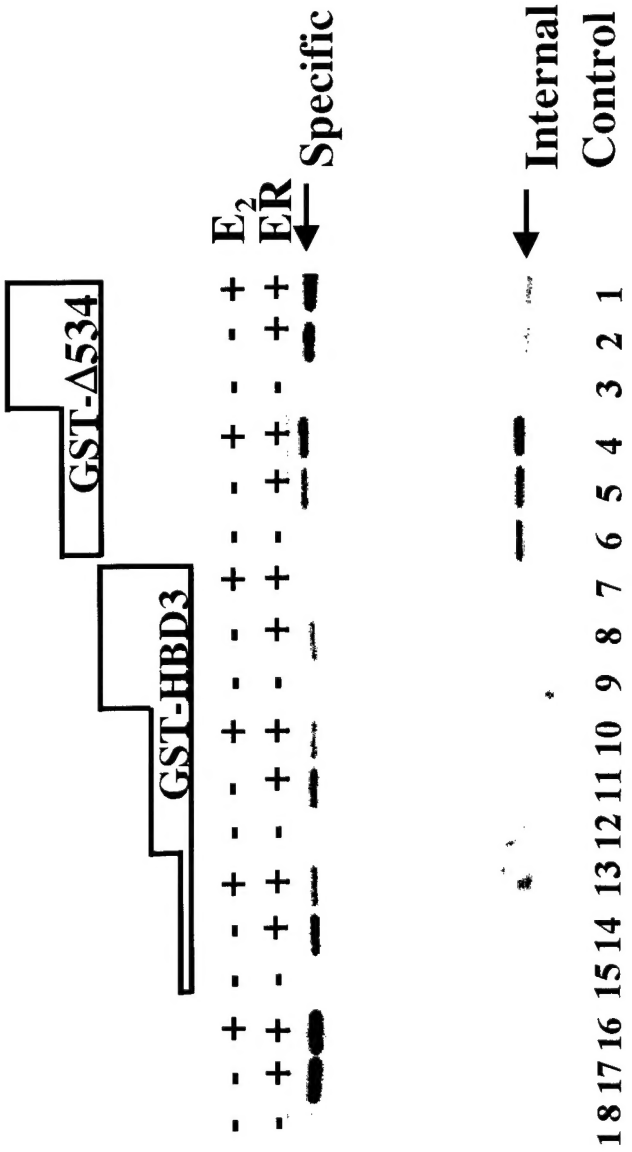

GST-Δ534
GST-HBD3
E2
ER
Specific
Internal
Control
1 8 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1

FIG. 1. **Stimulation of transcription *in vitro* by Partially purified baculovirus expressed human ER.** The transcriptional activity of ER was analyzed by *in vitro* transcription assay as described under "Experimental Procedures". Two hundred fmol of ER and 1 μM E_2 and 10 ng of the templates were used. Lane 1: the basal transcriptional activity of HeLa nuclear extract. Lane 2 and 3: the transcriptional activity of ER in the absence (lane 2) or presence (lane 3) of estradiol (E_2). The specific transcript (upper arrow) is from ERE-containing template pAdMLPERE3. The internal control transcript is from pAdMLPmERE template.

FIG. 2. **The effects of concentrations of templates on ER-mediated transcription *in vitro*.** The assay was performed as described in Figure 1 except that various amounts of templates (present in ng) were used.

FIG. 3. **The effects of concentrations of ER on ER-mediated transcription *in vitro*.** The assay was performed as described in Figure 1. Different amounts of ER were used.

FIG. 4. **ER-mediated transcription *in vitro* is hormone-dependent.** The assay was carried out in the presence of vehicle (lane 1 and 2), ICI 164,384 (4 to 400 μM) (lane 5 to 6), ICI182,780 (4 to 400 μM) (lane 10 to 12), 4-OH TAM (0.4 to 40 μM) (lane 16 to 18), E_2 (1 μM) (lane 3) or combination of ICI164,384 and E_2 (lane 7 to 9), combination of ICI182,780 and E_2 (lane 13 to 15) or combination of 4-OH TAM and E_2 (lane 19 to 21).

FIG. 5. **ER binds to ERE in a hormone-dependent manner.** Hormone-dependent DNA binding of ER was determined in the gel shift assay. ER was incubated with E2 (0.4 μM), 4-OH TAM (40 μM), ICI182,780 (40μM) or ICI164,384 (40 μM) or their combinations under the condition for transcription assay (Experimental Procedures).

FIG. 6. **The effect of GST-SRC-1 on ER-mediated transcription *in vitro*.** Increasing amount of GST-SRC-1 was added into the transcription system as indicated in the figure. The transcription assay was performed as described in Figure 1.

FIG. 7. **The effect of GST-HBD and GST-Δ534 on ER-mediated transcription *in vitro*.** GST-HBD and GST-Δ534 were described in text (16). Increasing amount of GST-HBD (400 fmol (lane 4 to 6), 800 fmol (lane 7 to 9), and 1600 fmol (lane 10 to 12)) and GST-Δ534 (800 fmol (lane 13 to 15) and 1600 fmol (lane 16 to 18) were introduced into the transcription mixture without (lane 1, 5, 7, 10, 14 and 17) or with (lane 2 and 3, 5 and 6, 8 and 9, 11 and 12, 14 and 15, 17 and 18) of 200 fmol of ER. Lane 3, 6, 9, 12, 15 and 18 contain E_2 (1 μM).

FINAL REPORT

Liu, H., Meyer, R., DiRenzo, J. and Brown, M. (1997) Estrogen receptor-Mediated transcription *in vitro*. Era of Hope.